The role of Rel$_{Mtb}$-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice

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Long-term survival of nonreplicating *Mycobacterium tuberculosis* (Mtb) is ensured by the coordinated shutdown of active metabolism through a broad transcriptional program called the stringent response. In Mtb, this response is initiated by the enzymatic action of Rel$_{Mtb}$ and deletion of *rel$_{Mtb}$* produces a strain (H37RvΔ*rel$_{Mtb}$*) severely compromised in the maintenance of long-term viability. Although aerosol inoculation of mice with H37RvΔ*rel$_{Mtb}$* results in normal initial bacterial growth and containment, the ability of this strain to sustain chronic infection is severely impaired. Significant histopathologic differences were noted in lungs and spleens of mice infected with H37RvΔ*rel$_{Mtb}$* compared with controls throughout the course of the infection. Microarray analysis revealed that H37RvΔ*rel$_{Mtb}$* suffers from a generalized alteration of the transcriptional apparatus, as well as specific changes in the expression of virulence factors, cell-wall biosynthetic enzymes, heat shock proteins, and secreted antigens that may alter immune recognition of the recombinant organism. Thus, Rel$_{Mtb}$ is critical for the successful establishment of persistent infection in mice by altering the expression of antigenic and enzymatic factors that may contribute to successful latent infection.

One billion people will be newly infected with *Mycobacterium tuberculosis* (Mtb) in the next two decades, leading to 35 million deaths worldwide (1). Although a fraction of those infected will immediately develop acute disease, the vast majority of individuals will contain the infection without any overt symptom other than conversion of their skin test response to purified protein derivative of tuberculin (PPD). These latent infections are characterized by an absence of bacterial replication. Even during active disease, nonreplicating organisms appear to be present, displaying a phenotypic drug resistance that necessitates the 6 month course of chemotherapy required to achieve a durable cure (2).

Successful containment of an Mtb infection typically coincides with the formation in the host tissues of granulomas that are aggregates of myeloid and lymphoid cells surrounding the infected macrophages. This cellular structure limits the dissemination of the bacteria while confronting them with an altered physical environment to which they must adapt to survive (3). Bacterial numbers within an intact, noncavitating granuloma in human tissues are not typically high, and replication is thought to be limited or nonexistent because of reduced availability of nutrients, oxygen, and iron (4, 5). *In vitro*, Mtb can persist for years in the absence of active replication after nutrient deprivation, whereas replenishing nutrients restores active metabolism and growth (6, 7). *In vivo* bacterial replication also slows during chronic infection, a result confirmed by recent studies of 16S rRNA levels in infected lungs (8). Only a restricted number of mycobacterial proteins have been identified that have been proposed to contribute to mycobacterial latency. These include isocitrate lyase, an enzyme involved in the utilization of host-derived lipid as a source of nutrients (9); α-crystallin, a chaperone involved in long-term stability of proteins (10); members of the PE-PGRS family of repetitive proteins whose function is unknown (11); and resuscitation promoting factor, proposed to operate as a "bacterial cytokine" (12). More global approaches, such as transcriptional profiling and proteomic analysis, have been applied to oxygen limitation and nutrient starvation (2, 13). However, only a single enzyme, isocitrate lyase, has been shown to be correlated directly with the ability of the organism to persist *in vivo* in animal models of latency.

When microorganisms encounter a nutrient-limited environment they slow their growth rate dramatically and reduce levels of rRNA, tRNA, and protein synthesis. Often, RNA polymerase activities are modified, the activity of transport systems is reduced, and metabolism of carbohydrates, amino acids, and phospholipids is decreased (14). Known as the stringent response, this broad alteration in metabolism is mediated by the accumulation of hyperphosphorylated guanine nucleotides, (p)ppGpp. These signaling molecules are synthesized by enzymatic transfer of pyrophosphate to GTP. The stringent response is reversed when environmental conditions become favorable and (p)ppGpp levels decrease. In *Escherichia coli*, (p)ppGpp acts by binding to the β-subunit of RNA polymerase and alters the expression of >80 different genes by affecting promoter specificity (15, 16). Recently the stringent response was shown to play a role in biofilm growth and adherence in *Listeria monocytogenes* (17), quorum sensing and cell-density-dependent gene expression in *Pseudomonas aeruginosa* (18), fruiting body development in *Myxococcus xanthus* (19), antibiotic production in *Streptomyces* (20), virulence regulation in *Legionella pneumophila* (21), and the development of antibiotic resistance (22).

Mtb has one dual-function enzyme, Rel$_{Mtb}$, for both (p)ppGpp synthesis and hydrolysis (23). This protein contains two distinct catalytic sites and is allosterically regulated by a complex of macromolecules consisting of the ribosome, tRNA, and mRNA (24). Inactivation of the gene encoding Rel$_{Mtb}$ produced a strain, H37RvΔ*rel$_{Mtb}$*, unable to synthesize (p)ppGpp on starvation and defective in long-term survival *in vitro* (25). To explore the impact of the stringent response on long-term survival during disease, we studied the behavior of the Rel$_{Mtb}$ mutant in a murine model of persistent tuberculosis.

Materials and Methods

Growth of Mycobacteria. Mtb H37Rv (American Type Culture Collection 27294), the H37RvΔ*rel$_{Mtb}$* mutant, and the complemented strain have all been described (25). Bacteria were grown

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in Middlebrook 7H9 broth (Difco), containing ADC (albumin [bovine, Fraction V], dextrose, and catalase) and Tween 80 (0.05% vol/vol), by using a rotary shaker (150 rpm) at 37°C. Bacteria were harvested at mid-logarithmic growth phase (OD_{650} 0.5–1.0) and frozen at −70°C before use in experimental infections of mice.

Infection of Mice. Five to eight week old female C57/BL6 mice were purchased from Taconic (Germantown, NY) and infected by aerosol as described (26). Colony-forming units (cfu) in lungs and spleens were assessed after homogenization in media by plating. Mice lungs and spleens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained for histology with either hematoxylin/eosin or Ziehl-Neelsen stain from American HistoLabs (Gaithersburg, MD).

RNA Isolation. Cells were grown to early-log phase (OD_{650} 0.3) in 7H9 broth containing ADC and Tween 80 in roller flasks. Cells were washed once with an equal volume of Tris-buffered saline with Tween (TBST) (50 mM Tris·HCl, pH 7.0/150 mM NaCl/ 0.05% Tween 80) and resuspended in an equal volume of prewarmed TBST and returned to the rolling incubator. At 0, 2, 4, and 6 h, 30-ml aliquots were removed for RNA extraction. RNA was isolated and purified as described (26).

Microarray Preparation. Fluorescently labeled cDNA was prepared from 4 μg of RNA, and all steps in preparation of the RNA and hybridization of microarrays were carried out as described (26). For microarray analysis, MADB TOOL GATEWAY software provided by the Center for Information Technology (http:// nciarray.nci.nih.gov) was used. Hybridizations were performed at least in duplicate by using RNA extracted from three independent experiments. All microarray experiments were repeated with reversed fluorescent labels. Signals were calculated from the median background-subtracted median spot intensities and were normalized by using the 50th percentile (median) and only included in the analysis if the values for both channels were at least one standard deviation above the average value of the negative control spots containing randomized hexamer oligonucleotides. Spots were omitted from further analysis if the calculated signal to background ratios in both were less than two. Two-fold changes in gene expression level in two or more separate arrays are reported. Selection criteria were ignored if the calculated signal in one channel was at least three times higher than the value of the negative control spots plus one standard deviation.

Results

Failure to Induce the Stringent Response Attenuates Mtb for Persistence in Murine Tissues. To examine the effect of loss of Rel_{Mtb} on bacterial growth and persistence, C57/BL6 mice were challenged by aerosol infection with 50–100 organisms of wild-type H37Rv, H37RvΔrel_{Mtb}, or the complemented strain H37RvΔrel_{Mtb} attB::rel_{Mtb}. Two to 5 weeks postinfection, replication of the Rel_{Mtb}-deficient strain was largely indistinguishable from either the wild-type or the complemented strain in both mouse lungs and spleen (Fig. 1). Between 5 and 7 weeks, the H37RvΔrel_{Mtb} strain began to lose viability relative to the two strains capable of engaging the stringent response. By 38 weeks postinfection the H37RvΔrel_{Mtb} strain had dropped to levels 500-fold lower than H37Rv and the complemented strain in both lungs (Fig. 1a) and spleens (Fig. 1b).

**Loss of Rel_{Mtb} Reduces Disease-Associated Weight Loss and Gross Pathology.** To examine the effect of loss of Rel_{Mtb} on disease progression, infected mice were evaluated for weight gain and organ pathology over time. By 21 weeks postinfection a difference in body weight was noted among groups of mice infected
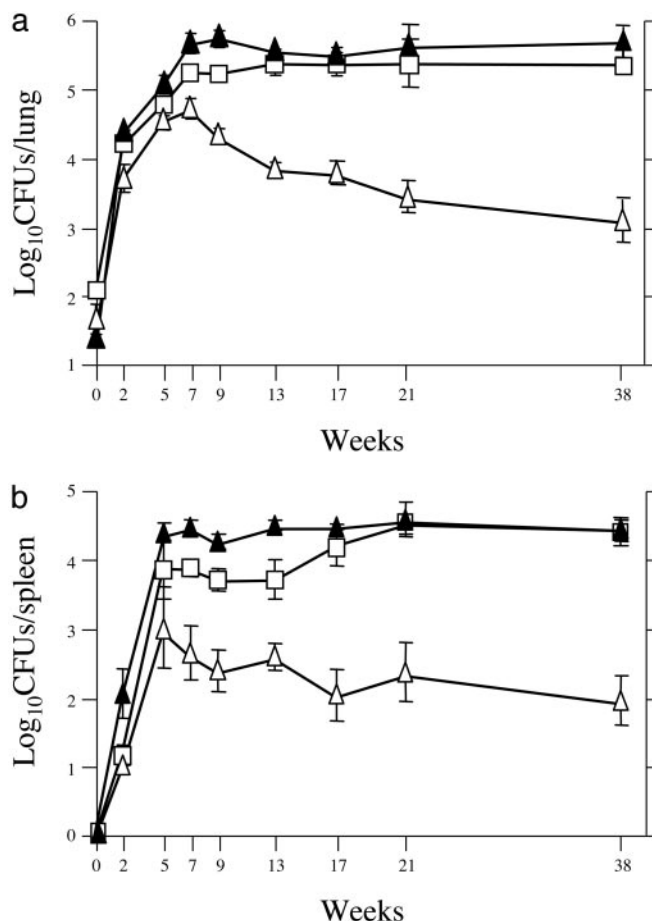
Fig. 1. Growth of Rel_{Mtb}-deficient H37Rv in the lungs (*a*) and spleens (*b*) of low-dose aerosol-infected C57BL/6 mice. In both graphs, open squares represent the colony-forming units (cfu) of wild-type H37Rv and open triangles represent the cfu of the RelMtb-defective strain H37RvΔrel_{Mtb}. Filled triangles represent the complemented strain H37RvΔrel_{Mtb}attB::rel_{Mtb}. These data are representative of three independent experiments.

with the different Mtb strains. Mice infected with H37RvΔrel_{Mtb} weighed 8% more on average than mice in the two control groups ($P = 0.039$; Fig. 2a). In addition, mice infected with H37RvΔrel_{Mtb} had much more adipose tissue surrounding internal organs compared with mice from the two other groups (data not shown). Gross examination of the lungs of infected mice at 5 weeks postinfection revealed the presence of small visible granulomas (not shown). In the lungs of mice infected with H37RvΔrel_{Mtb}, only very few of these granulomas increased in size over time. In the lungs of mice infected with the two other strains, multiple granulomas increased in size during disease progression. Representative lungs from mice infected with the three different Mtb strains at 21 weeks postinfection are shown in Fig. 2b. The majority of H37RvΔrel_{Mtb}-induced granulomas were smaller than the granulomas seen in the lungs of mice infected with either strain containing an intact Rel_{Mtb} protein. H37RvΔrel_{Mtb}-infected mice had spleens that were significantly smaller than those of H37Rv-infected mice (Fig. 2c).

Loss of Rel_{Mtb} Reduces Mtb-Induced Histopathology. Histologic examination of the lungs of mice 15 weeks after infection revealed striking differences in the granulomatous response among the three groups of mice. Multiple large granulomas were present throughout the lungs of H37Rv and H37RvΔrel_{Mtb} attB::rel_{Mtb}-infected mice occupying as much as one-third of the
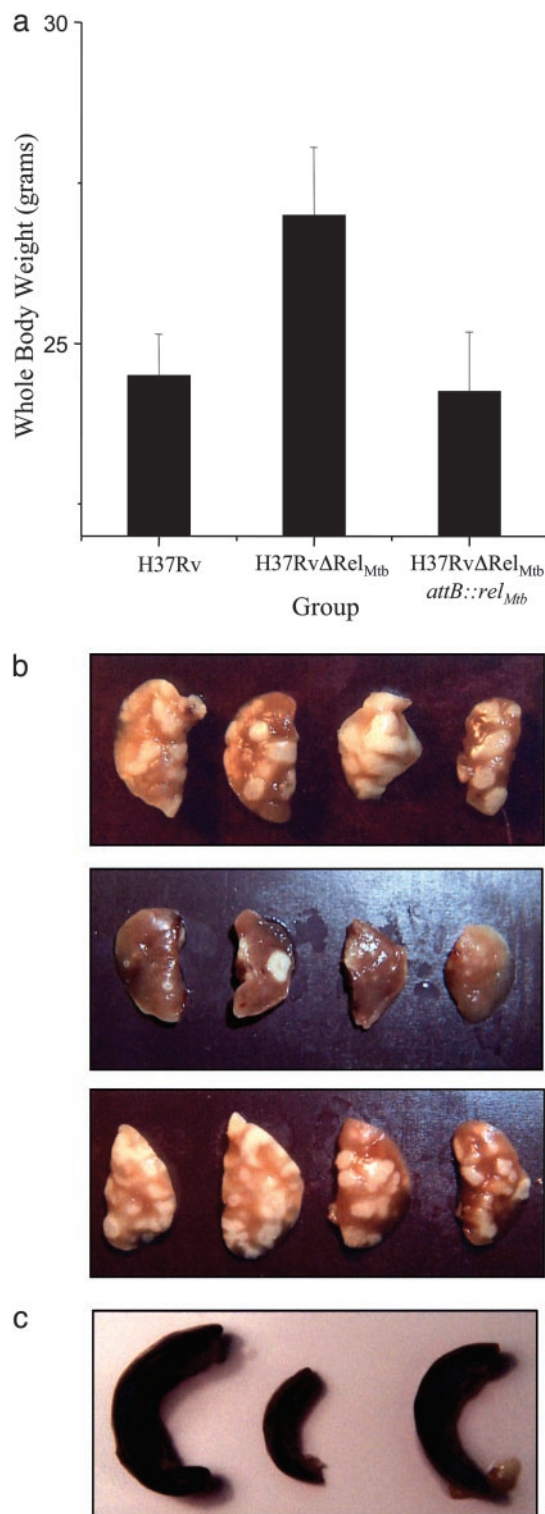
Fig. 2. Weight gain (*a*) and gross pathology in lungs (*b*) and spleens (*c*) of aerosol-infected C57BL/6 mice. (*a*) Mean ± SD for the weights of groups of four mice evaluated 15 weeks after infection. (*b*) The lungs of mice infected with wild-type H37Rv (*Top*), H37RvΔ*rel_Mtb* (*Middle*), and H37RvΔ*rel_Mtb*attB::*rel_Mtb* (*Bottom*). (*c*) Spleens of mice infected with wild-type H37Rv (left), H37RvΔ*rel_Mtb* (center), and H37RvΔ*rel_Mtb*attB::*rel_Mtb* (right).

total lung area (Fig. 3 *a* and *c*). The remaining lung parenchyma showed extensive edema, mononuclear leukocyte infiltration of the alveolar septi, and significant compensatory emphysema

(Fig. 3 *g* and *i*). In contrast, mice infected with the H37RvΔ*rel_Mtb* strain maintained almost normal lung architecture, and the few granulomas found in the lungs of these mice were significantly smaller with similar cellular composition (Fig. 3 *b*, *e*, and *h*). Granulomas showed extensive lymphocytic aggregates, as well as large numbers of foamy macrophages. Ziehl-Neelsen staining revealed the presence of acid-fast bacilli in the granulomas of mice infected with both the wild-type and complemented strains but not in the H37RvΔ*rel_Mtb*-infected lung samples.

At 38 weeks postinfection, lesions in the lungs of mice infected with wild-type and complemented strains are extensive and occupy almost the entire parenchyma (Fig. 4). The H37RvΔ*rel_Mtb*-infected lungs were also heavily infiltrated; however, the infiltrates were less cellular, with very few lymphoid aggregates and more residual lung parenchyma (Fig. 4 *b* and *e*). At high magnification, differences in the morphology of the macrophages among the three groups of mice were apparent (Fig. 4 *d–f*). Whereas the macrophages in the granulomas of the wild-type and complemented-strain-infected mice were clearly foamy, macrophages in the lungs of the H37RvΔ*rel_Mtb*-infected mice were more epithelioid with few (if any) foamy vacuoles.

Rel_Mtb Down-Regulates the Mycobacterial Translational Apparatus in Response to Starvation. To identify specific gene products involved in determining the *in vivo* phenotype of the H37RvΔ*rel_Mtb* strain, we compared the transcriptional profile of this strain with that of the wild-type strain during nutrient starvation. This comparison revealed that more than one-fourth of the genome (1,049 genes; see Data Set 1, which is published as supporting information on the PNAS web site, www.pnas.org) was differentially expressed between the two strains under these conditions. When gene expression was analyzed during nutrient-sufficient conditions many of these differences remained, suggesting that there are extensive metabolic alterations in the mutant strain lacking (p)ppGpp. Changes in the transcriptional profile induced by 6 h of starvation in each strain separately revealed that 575 genes were differentially regulated in the wild-type organism compared with only 341 in the mutant. From this we could identify the Rel_Mtb regulon by selecting for genes that were either induced (69) or repressed (90) in the wild-type strain on nutrient limitation but whose expression was not altered on starvation of the mutant strain. These 159 genes comprise the nominal Mtb gene set under direct control of the stringent response (Data Set 1). H37Rv, but not H37RvΔ*rel_Mtb*, globally down-regulated the cellular translational apparatus consistent with Rel-mediated effects in other bacteria (14). Of 58 ribosomal proteins, 54 were down-regulated in wild-type H37Rv on nutrient starvation along with 16 other genes involved in various aspects of protein synthesis and 5 genes involved in transcription.

H37RvΔ*rel_Mtb* Shows Differential Expression of Known Secreted Antigens and Virulence Factors. To understand the defect in persistence of the H37RvΔ*rel_Mtb* mutant we examined the list of differentially expressed genes between this strain and the wild-type organism. Among the genes whose expression was significantly altered between the two strains were a number of virulence genes that have been previously linked with defects in persistence of the organism, such as the anaerobic nitrate reductase components *narH* and *narI* (Table 1). The list of differentially expressed genes also includes 11 enzymes involved in remodeling of the mycobacterial cell wall, 3 polyketide synthase genes, and 3 separate operons encoding proteins proposed to play a role in macrophage cell entry (*mce* genes).

There was also a large set of known mycobacterial antigens differentially expressed between the two strains such as *groEL*2 and *groES*, the 19-kDa antigen, and six members of the PE/PE-PGRS protein family. Secreted antigens of the culture filtrate
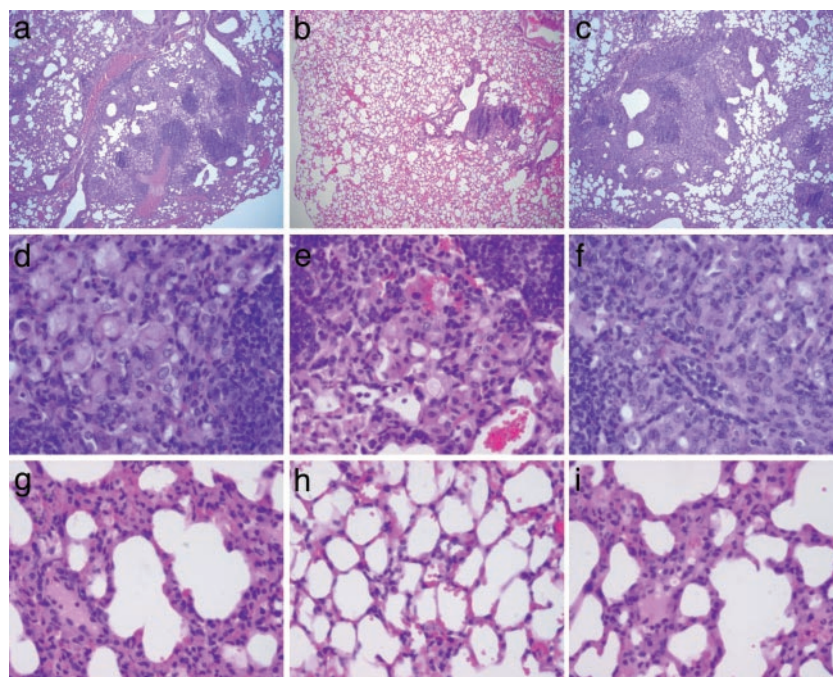
Fig. 3. Histopathology of the lungs of aerosol-infected mice 15 weeks after exposure. (*a*, *d*, and *g*) Wild-type H37Rv. (*b*, *e*, *h*) H37RvΔ*rel*$_{Mtb}$. (*c*, *f*, and *i*) H37RvΔ*rel*$_{Mtb}$attB::*rel*$_{Mtb}$. (*a–c*) ×4 magnification. (*d–f*) Granulomatous tissue at ×40 magnification. (*g–i*) Uninvolved lung tissue at ×40 magnification.

found to be dysregulated included ESAT-6, the antigen 85 complex, Mpt83, and Cfp7. Many of these antigens appear to be controlled by RelA$_{Mtb}$ (regulated in the wild type on starvation but unchanged in the H37RvΔ*rel*$_{Mtb}$ mutant), including the 19-kDa major antigen and most of the antigen 85 complex.

Independent confirmation of the microarray data were performed by Western blot analysis of lysates derived from H37Rv and H37RvΔ*rel*$_{Mtb}$ grown to stationary phase in rich media. Primary antibodies specific for the Eis protein (Rv2416c) (27) demonstrated accumulation of this protein in the Δ*rel*$_{Mtb}$ strain but not in the wild-type strain (data not shown). In addition, induction or repression of nine randomly selected genes was confirmed by quantitative RT-PCR (Data Set 1).

Discussion

There is compelling evidence that deprivation of nutrients produces bacilli with similar qualities to bacilli *in vivo*. Mtb isolated directly from lung lesions have altered morphology and reduced acid-fast staining similar to Mtb starved in distilled water or PBS (7). Bacilli starved in this way respire much more slowly until returned to rich media and can survive up to 2 years with no source of nutrients (6, 28). Although this probably does not reflect the situation in granulomas that necrose and cavitate, subsequently shedding bacilli into sputum, it may reflect the majority of noncavitating granulomas that contain nonreplicating bacteria (29). The results presented here support this model because eliminating the major bacterial mechanism for starvation adaptation impairs persistence in the host (25). Thus, nutrient deprivation may be a major component of successful host containment of a mycobacterial infection.

Recently a whole genome microarray analysis of H37Rv under starvation conditions was reported (2). Considering the differences in experimental protocols used, concordance between
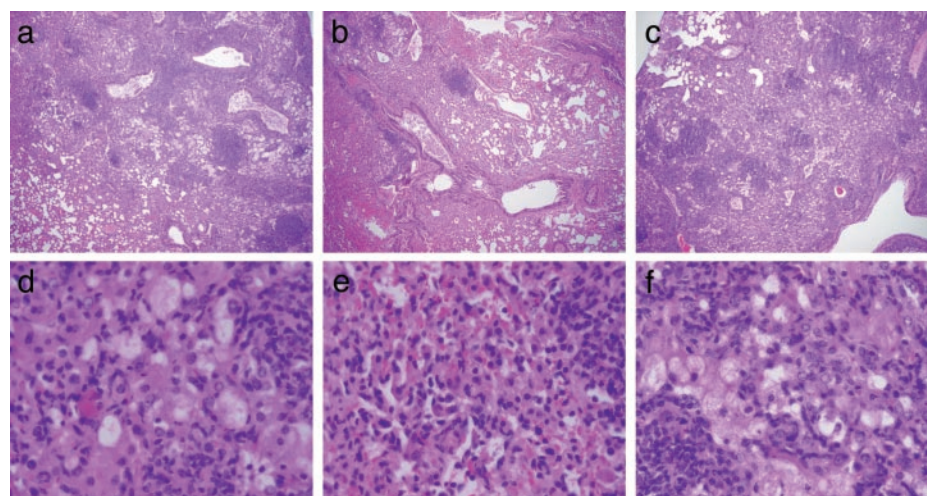
Fig. 4. Histopathology of the lungs of aerosol-infected mice 38 weeks after infection. (*a* and *d*) Wild-type H37Rv. (*b* and *e*) H37RvΔ*rel*$_{Mtb}$. (*c* and *f*) H37RvΔ*rel*$_{Mtb}$attB::*rel*$_{Mtb}$. (*a–c*) ×4 magnification. (*d–f*) Granulomatous tissue at ×40 magnification.

Table 1. Differentially expressed genes by comparative microarray

<div align="center">Virulence factors and cell wall remodeling</div>

Higher expression in wild type

Rv0016c	*pbpA*, probable penicillin-binding membrane protein
Rv0169, Rv0170, Rv0171, Rv0172, Rv0173, and Rv0174	mce1 operon, cell invasion
Rv1162	*narH*, Nitrate reductase b chain
Rv1164	*narI*, Nitrate reductase g chain
Rv1181, Rv1182	*pks4*, *papA3*
Rv1477	Putative invasion protein
Rv1527c	*pks5*
Rv1916	*aceAb*, Ic1 b module
Rv1967	*mce3B*, mce3 operon, cell invasion
Rv2151c	*ftsQ*, ingrowth of wall at septum
Rv2152c	*murC*, peptidoglycan synthesis
Rv2153c	*murG*, peptidoglycan synthesis
Rv2154c	*ftsW*, membrane protein (shape determination)
Rv2163c	pbpB, probable penicillin-binding membrane protein
Rv2244, Rv2245, and Rv2246	*acpM*, *kasA*, and *kasB*, fas II system
Rv2524c	*fas*
Rv2583c	*relA*, ppGpp synthetase I
Rv3414c	*sigD*
Rv3494c, Rv3495c, Rv3496c, and Rv3497c	mce4 operon, cell invasion

Higher expression in relA mutant

Rv0440	*groEL2*, heat shock protein 65
Rv0644c	*mmaA2*, methoxymycolic acid synthase 2
Rv0899	*ompA*, outer membrane protein
Rv2416c	*eis*
Rv2947c	*pks15*
Rv3416	*whiB3*, transcriptional regulator
Rv3418	*groES*, 10-kDa chaperone

<div align="center">Antigens and secreted proteins</div>

Higher expression in wild type

Rv0129c	*fbpC*, mycolyltransferase 85C
Rv0288	*cfp7*
Rv0291	Secreted protease
Rv0934	*pstS1*, major antigenic protein, PstS phosphate transport system
Rv1368	*lprF*, lipoprotein
Rv1884c	*rpfC*
Rv2031c	*hspX*, 14-kDa antigen, heat shock protein Hsp20 family
Rv2253	Probable secreted protein
Rv3019c	Similar to Esat6
Rv3144c, Rv1646	PE/PPE family members
Rv3763	*lpqH*, 19-kDa (major antigenic protein)
Rv3875	*esat6*, early secretory antigen target

Higher expression in relA mutant

Rv0203	Possible exported protein, has signal peptide
Rv0867c	Probable exported protein
Rv0947c	Probable mycolyl transferase
Rv1288	Similarity to antigen 85B
Rv1790, Rv2430c, Rv2487c, Rv3872, Rv2519, and Rv1172c	PE/PPE/PE-PGRS family members
Rv1886c	*fbpB*, mycolyl transferase, antigen 85B
Rv2290	*lppO*, lipoprotein
Rv2301	*cfp25*, cutinase, secreted protein
Rv2518c	*lppS*, lipoprotein
Rv2784c	*lppU,* lipoprotein
Rv2873	*mpt83*
Rv3803c	*fbpD,* mycolyl transferase, antigen 85C
Rv3804c	*fbpA,* mycolyl transferase, antigen 85A

these data and our data set was high. The loss of Rel$_{Mtb}$, and the corresponding lack of (p)ppGpp in the cell, was found to induce major shifts in metabolism by microarray analysis, even during logarithmic growth. We identified 159 genes that were regulated by (p)ppGpp under starvation, comparable to that observed for the RelA of other bacteria (>80) (30).

In addition to playing a role in coordinating slowdown of bacterial metabolism, Rel$_{Mtb}$ affects many genes that have been implicated in, or can plausibly be linked to, persistence or virulence of Mtb. Multiple genes within three different operons encoding macrophage cell entry (*mce*) proteins show Rel$_{Mtb}$-dependent enhanced expression (31). Both the ability of Mtb to use nitrate as a terminal electron acceptor in respiration and the ability of Mtb to engage the glyoxylate shunt enzyme isocitrate lyase have been previously shown to play a role in virulence or persistence, and genes encoding elements of both these systems

are regulated or affected by Rel$_{Mtb}$ (9, 32). The significance of the up-regulation of AceAb is unknown because *icl* is up-regulated in both wild-type and mutant strains (Data Set 1). Extensive alterations in expression levels of cell-wall biosynthetic genes are evident in the absence of Rel$_{Mtb}$, although control of these genes appears to be more complex than by Rel$_{Mtb}$ alone. Remodeling of the mycobacterial cell wall has been proposed to accompany both macrophage infection and the transition to stationary phase (33, 34). Several polyketide synthases, whose enzymatic products may play a role in modulating the host immune response, are also Rel$_{Mtb}$-affected. Finally, Mtb contains several protein families composed largely of repeated amino acid motifs that have been proposed to play a role in antigenic variation among different clinical isolates (the PE/PPE/PE-PGRS proteins) (35, 36). Eight of these proteins, proposed to play a role in granuloma formation and persistence, are affected by Rel$_{Mtb}$ (11).

Our results also suggest the surprising possibility that some of the most potent mycobacterial antigens may be directly under the control of intracellular (p)ppGpp levels. Two of the three components of the antigen 85 complex (and another mycolyl-transferase, Rv0947c) appear in the list of 159 genes that lose starvation responsiveness in the mutant strain. Because these proteins have been shown to play a role in cell wall construction, coupling their expression to nutrient availability through the stringent response makes metabolic sense (37, 38). In addition to their biochemical role in cell wall formation, proteins of the antigen 85 complex have been shown to drive naïve human T cells to differentiate toward a Th1 phenotype in *in vitro* models (39). When antigen 85A is injected into C57BL/6 mice, an elevated IFN-γ response is generated that is protective against i.v. challenge with wild-type Mtb (40). Thus, the relative over-expression of antigen 85A and C in the mutant strain may provide for enhanced clearance because of increased host recognition of this protein. A large number of secreted culture-filtrate proteins are also altered in expression in the mutant strain, including the potent T cell antigen *esat*6 (Rv3875), which is relatively underexpressed in the mutant. Deletion of this gene from *Mycobacterium bovis* produces a strain that is attenuated in guinea pigs (41). The 19-kDa antigen *lpqH*, which has been shown to inhibit cytokine secretion, decrease antigen presentation by macrophages, and promote macrophage apoptosis, and is a target for T cell recognition, appears to be primarily regulated by Rel$_{Mtb}$ (42).

These experiments show that the Mtb stringent response is critical for bacterial survival under persistent infection conditions in mice. The direct relevance of the stringent response for the maintenance of chronic, asymptomatic infection in humans remains to be established. The Rel$_{Mtb}$ regulon, however, provides a critical list of potential targets ideally suited for developing novel treatments for latent infections or for shortening the course of antituberculosis chemotherapy (2). Rel$_{Mtb}$-regulated genes also provide a rich source of potentially specific diagnostic markers that may be predictive of successful tuberculosis chemotherapy.

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